AD________________

GRANT NUMBER DAMD17-96-1-6274

TITLE: Molecular Interactions of Bcl-2 Family Members in Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Gabriel Nunez, M.D.

CONTRACTING ORGANIZATION: University of Michigan
Ann Arbor, Michigan 48103-1274

REPORT DATE: September 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1997	Annual (1 Sep 96 - 31 Aug 97)

4. TITLE AND SUBTITLE
Molecular Interactions of Bcl-2 Family Members in Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-96-1-6274

6. AUTHOR(S)
Gabriel Nunez, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Michigan
Ann Arbor, MI 48103-1274

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

The treatment of cancer with chemotherapy or radiation therapy is limited by the emergence of tumor cells resistant to these therapies. This resistance limits our ability to successfully treat these neoplasms. A major genetic event that occurs in the genesis and/or progression of cancer is alteration in the pathway of apoptosis. Recent studies indicate that the susceptibility of cancer cells to therapy-induced death is controlled in part by genes that regulate the apoptosis pathway. Of these, bcl-2 and bcl-x are expressed in a wide variety of cancer cells and encode products, bcl-2 and bcl-x_L, that repress the apoptotic mechanism. In order to assess the function of bcl-2 and bcl-x in maintaining the survival of cancer cells, an adenoviral vector that expresses bcl-x_S, a dominant inhibitor of bcl-2 and bcl-x_L was constructed. In the absence of exogenous signals, the bcl-x_S adenovirus killed cancer cells by apoptosis including primary breast carcinomas. We postulate that apoptotic signals are constitutively expressed in proliferating cancer cells, although repressed by members of the bcl-2 family of proteins. In this proposal, we propose experiments (i) to determine the mechanism involved in bcl-x_S-mediated apoptosis of cancer cells using the bcl-x_S adenovirus to dissect molecular interactions of the bcl-2 regulatory pathway and (ii) to characterize cellular proteins that interact with bcl-x_S using biochemical and genetic approaches. The studies outlined in this proposal should provide novel insight into the apoptosis pathway and lead to alternative therapeutic strategies for the treatment of cancer.

14. SUBJECT TERMS Breast Cancer

15. NUMBER OF PAGES
10

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

N/A Where copyrighted material is quoted, permission has been obtained to use such material.

N/A Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

X Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

[signature] 9-25-97

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

Cancer is the result of multiple genetic events, including activation of oncogenes and inactivation of tumor suppressor genes. The protein products of the former are often mitogens, whereas the products of the latter suppress proliferation. It is becoming increasingly apparent that tumor suppressor genes like p53 function in part by activating an apoptotic death pathway. In addition, certain oncogenes such as bcl-2 appear to contribute to tumor development primarily by promoting abnormal cell survival via an apoptosis inhibitory signal. Thus, disruption of the apoptosis pathway appears integral to many malignancies including breast cancer. Furthermore, treatment of cancer with chemotherapy or radiation therapy is limited by the emergence of tumor cells resistant to these therapies. This resistance limits our ability to successfully treat these neoplasms.

The product of the *bcl-2* gene, which is activated via translocation in B-cell lymphomas, is an important modulator of apoptosis in many types cell lineages and tissues (1). *Bcl-2* encodes an integral-membrane protein originally found to inhibit apoptosis of hematopoietic cell lines following growth factor deprivation, and later to prevent or delay apoptosis induced by a large variety of stimuli including γ-irradiation, glucocorticoids, heat shock and many chemotherapeutic drugs (2-5). In addition to Bcl-2, a structurally related protein Bcl-x_L, has been identified by Craig Thompson's and our laboratory (6-7). Bcl-x_L exhibits different regulation than Bcl-2, but like Bcl-2 inhibits apoptosis induced by a variety of signals (6-11). The biochemical mechanism by which Bcl-2 and Bcl-x_L block apoptosis is not yet clear. However, Bcl-2 is known to interact with several cellular proteins (12-17). Some of these proteins, for example Bax, Bad, and Bak, share significant amino acid homology with Bcl-2 and Bcl-x_L and are considered members of the Bcl-2 family (18). Bax, Bad and Bak appear to negatively affect Bcl-2 and/or Bcl-x_L function through protein-protein interactions (13-15). In cancer, one of the most common abnormalities is deregulated expression of the Bcl-2 oncoprotein. Up to 60% of cancers originating from many organs including breast overexpress a member of the Bcl-2 family of proteins that inhibits apoptosis (19-23).

A major genetic event that occurs in the genesis and/or progression of breast carcinoma involves alterations in the pathway of apoptosis. We hypothesize that one of these is the functional deregulation of the Bcl-2 family of proteins and that expression of these proteins plays a critical role in the maintenance of breast cancer cells and resistance of tumor cells to therapy-induced apoptosis. To determine the role of the Bcl-2 family of proteins in maintaining cancer cell viability, we constructed a recombinant adenovirus vector that expresses Bcl-x_S, a dominant inhibitor of these proteins. Even in the absence of an exogenous apoptotic signal, this recombinant virus specifically and efficiently activates apoptosis in human carcinoma cells arising from multiple organs including breast, colon, stomach and sympathetic nervous tissue (24). Based on these results, we hypothesize that apoptotic signals are constitutively expressed in proliferating cancer cells and perhaps in normal cells, although repressed by members of the Bcl-2 family of proteins. In this proposal, we proposed studies (i) to determine the mechanism involved in Bcl-x_S-mediated apoptosis using the *bcl-x_S* adenovirus to dissect molecular interactions of the Bcl-2 regulatory pathway, and ii) to characterize cellular proteins that interact with Bcl-x_S using biochemical and genetic approaches. The studies outlined in this proposal may provide novel insight into the apoptosis pathway and lead to alternative therapeutic strategies for the treatment of breast cancer and other malignancies.

BODY OF THE ANNUAL REPORT

Technical Objective #1: Further characterization of the interaction of Bcl-x_S with Bcl-2, Bcl-x_L and Bax in breast cancer cells (Tasks 1.1 and 1.2).

We presented evidence in the original proposal that MCF-7 breast tumor cells and primary breast carcinoma cells undergo apoptotic cell death after exposure to a *bcl-x_S* adenovirus vector. Our hypothesis is that inactivation of Bcl-2 or Bcl-x_L by Bcl-x_S unleashes endogenous death signals leading to execution of the apoptotic program. We proposed experiments to examine molecular interactions of the Bcl-2 family members following expression of Bcl-x_S protein, but prior to morphological/biochemical cell death, in order to define those interactions which may be created or destroyed as the apoptotic program is activated.

In these experiments, cancer cells that express stably FLAG-tagged Bcl-2 or Bcl-X_L were infected with the *bcl-x_S* or control β-galactosidase adenovirus vector. Twenty hours later the infected cells were cultured in media containing ^{35}S-methionine-cysteine for 4-5 hrs to label newly synthesized proteins in infected cells. Cells were then lysed and protein was immunoprecipitated with anti-FLAG antibody in a buffer containing non-ionic detergents. After immunoprecipitation, the protein complexes were disrupted by heating in the presence of 1% SDS. The supernatant was then diluted and reimmunoprecipitated using anti- Bcl-X or control antibody. Using this approach, it was difficult to detect interactions between Bcl-X_S and Bcl-2 or Bcl-X_L due to the low sensitivity of the assay. To overcome this problem, we performed sequential Immunoprecipation/Western-blot analysis of the same lyates. In this type of experiment, cellular lysates were immunoprecipated with anti-FLAG and protein complexes were resolved by SDS-PAGE, transfered to nitrocellulose, and immunoblotted with anti-Bcl-X antibody. These experiments showed that Bcl-X_S interacts with Bcl-X_L but not with Bcl-2.

We determined next if Bcl-X_S expression could alter the interaction of Bcl-2 or Bcl-X_L with BAX in SHEP cells. In these experiments, FLAG-tagged-Bcl-2/Bax or Bcl-X_L/Bax complexes were immunoprecipitated with anti-FLAG in the presence or absence of Bcl-X_S, and the amount of bound BAX was determined by immunoblotting with anti-BAX antibody. The results showed that Bcl-X_S displaces BAX from Bcl-2 and Bcl-X_L after normalization for equal amounts of BAX. We could not determine if Bcl-X_S could alter the amount of BAK, another partner of Bcl-2 and Bcl-X_L because the cells did not express detectable levels of endogenous BAK.

In additional experiments, we have performed extansive mutagenesis of the Bcl-Xs protein to determine the regions of Bcl-X_S required for its interaction with Bcl-X_L and those involved in breast cancer cell killing. We have expressed the Bcl-Xs mutants in cells in vivo and are currently performing functional and biochemical analysis of the Bcl-xs mutants.

Recent experiments have suggested that Bcl-2 and Bcl-X_L regulate apoptosis by interacting with and inhibiting caspases (death proteases) through a mammalian homologue of the *C. Elegans* CED-4 protein (25-26). Consequently, we are exploring the possibility that Bcl-X_S promotes apoptosis by interfering with the ability of Bcl-2 and Bcl-X_L to regulate caspase activity. In preliminary experiments, we have found that Bcl-X_S can form a multimeric complex with Bcl-X_L and *C. Elegans* CED-4. Future experiments are aimed at exploring functional and biochemical interactions of Bcl-X_S with Bcl-X_L, CED-4 and mammalian caspases.

Technical Objective #2: Further characterization and purification of p15, a cellular protein that interacts with Bcl-x_S.

A mechanism that might explain the apoptosis-promoting activity of Bcl-x_S is through binding to an upstream activator or a downstream effector of Bcl-2/Bcl-x_L mediated survival. In preliminary results, we provided evidence in the original application that Bcl-x_S interacts with a cellular protein, p15, in cancer cells infected with the *bcl-x_S* adenovirus. The significance of the Bcl-x_S-p15 interaction was unclear. p15 could represent a death effector which is activated by the expression of Bcl-x_S. Alternatively, p15 could be a cellular protein required for survival whose activity is inhibited by the Bcl-x_S interaction. Clearly, biochemical characterization of p15 and isolation of the p15 cDNA was needed to further assess the role of p15 in Bcl-x_S-mediated apoptosis.

Biochemical Purification and N-Terminal Microsequencing of p15 (Task 2.1) We purified p15 from preparative gels and submitted the material for microsequencing. Sequencing analysis revealed that the N-terminal sequence of p15 is identical to that of a region of Bcl-2, amino acids that are part of the flexible loop region (27). Thus, p15 represents a cleavage product of Bcl-2. Given these results we are not pursuing any longer the characterization of the protein band termed p15.

Genetic Screen for Bcl-x-Binding Proteins using the Two-Hybrid Yeast Assay (Task 2.2). To search for Bcl-x-interacting proteins, we screened placenta and brain cDNA libraries using Gal4-Bcl-x_L and Gal4-Bcl-x_S as "baits" in the yeast two-hybrid assay. In the first screen using the GAL4-Bcl-x_L bait, fifty six positive clones were identified that interacted with Gal4-Bcl-x_L. Forty one cDNAs encoded Bad, a Bcl-2 family member recently isolated by binding to Bcl-2. Ten cDNAs encoded Bcl-2 which is known to bind Bcl-X_L in the two-hybrid system. The nucleotide sequences of four cDNAs were novel in that they did not reveal significant homology to any known gene or translated products in the databases. Three of these novel cDNAs encoded the same gene which we have named *harakiri.* The same gene was identified as an interacting partner of Bcl-2 (28). *Harakiri* functions as a regulator of Bcl-2 and Bcl-X_L and apoptotic cell death in mammalian cells. The *hrk* product (HRK) does not exhibit significant homology to Bcl-2 or Bcl-X_L and lacks the conserved BH1 and BH2 domains which are shared by Bcl-2 family members. Significantly, HRK physically interacts with anti-apoptosis proteins Bcl-2 and Bcl-X_L but not with death-promoting Bcl-2 members such as Bax and Bak. Expression of HRK induces rapid onset of cell death in mammalian cells including breast cancer cells. Importantly, the death-promoting activity of HRK is repressed by Bcl-2 and Bcl-X_L suggesting that HRK is a common target for the anti-apoptosis proteins Bcl-2 and Bcl-X_L. Details of these results have been recently published (Inohara *et al.* The EMBO J. 16:1686-1694, 1997).

CONCLUSIONS

The studies which we have performed in the past year are important in that they have provided information regarding the mechanism by which the Bcl-X_S adenovirus kills tumor cells. Particularly revealing was the interaction of Bcl-X_S with Bcl-X_L but not with Bcl-2 suggesting that Bcl-X_S functions at least in part through another regulator. A candidate for this regulator is a mammalian homologue of the *C. Elegans* protein CED-4. Through a genetic screen in yeast, we have identified and characterized HRK, a novel protein that interacts with Bcl-2 and Bcl-X_L and induces apoptosis of cancer cell lines including breast tumor cells. Although the mechanism of the phenotype remains unclear, these results suggest that proteins of the Bcl-2 survival pathway that are targeted by Bcl-X_S may be required for the terminal differentiation of mammary epithelium induced by pregnancy.

REFERENCES

1. Vaux, D.L., Cory, S., and Adams, J.M. (1988). Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature, 335:440-442.
2. Nuñez, G., London, L., Hockenbery, D., Alexander, M., McKearn, J.P., and Korsmeyer, S.J. (1990). Deregulated Bcl-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. J. Immunol., 144:3602-3610.
3. Sentman, C.L., Shutter, J.R., Hockenbery, D., Kanagawa, O., and Korsmeyer, S.J. (1991). Bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. Cell, 67:879-888.
4. Miyashita, T. and Reed, J.C. (1992). bcl-2 gene transfer increases relative resistance of S49.1 and WEH17.2 lymphoid cells to cell death and DNA fragmentation induced by glucocorticoids and multiple chemotherapeutic drugs. Cancer Res., 52:5407-5411.
5. Dole, M., Nuñez, G., Merchant, A.K., Maybaum, J., Rode, C.K., Bloch, C.A., and Castle, V.P. (1994). Bcl-2 inhibits chemotherapy-induced apoptosis in neuroblastoma. Cancer Res., 54:3253-3259.
6 Boise, L.H., González-Garcia, M., Postema, C.E., Ding, L., Lindsten, T., Turka, L.A., Mao, X., Nuñez, G., and Thompson, C.B. (1993). *bcl-x*, a *bcl-2* related gene that functions as a dominant regulator of apoptotic cell death. Cell, 74:597-608.
7. González-García, M., Perez-Ballestero, R., Ding, L., Duan, L., Boise, L.H., Thompson, C.B., and Nuñez, G. (1994). *bcl-x*$_L$ is the major *bcl-x* mRNA form expressed during murine development and its product localizes to mitochondria. Development, 120:3033-3042.
8. Gottschalk, A.R., L.H. Boise, C.B. Thompson, and J. Quintans. (1994). Identification of immunosuppressant-induced apoptosis in a murine B-cell line and its prevention by bcl-x but not bcl-2. Proc. Natl. Acad. USA, 91:7350-7354.
9. Fang W., Rivard J. J., Ganser J. A., LeBien T. W., Nath K. A., Mueller D. L., and Behrens T. W. (1995). Bcl-x$_L$ rescues WEHI-231 B lymphocytes from oxidant-mediated death following diverse apoptotic stimuli. J. Immunol., 155:66-75.
10. Gonzalez-Garcia, Garcia I., O'Shea S., Ding L., Boise L., Thompson C. B., and Nuñez G. (1995). *bcl-x* is expressed in embryonic and postnatal nervous tissues and functions to prevent neuronal cell death. Proc. Natl. Acad. Sci. USA, 92:3404-3408.
11. Grillot D.M. A. , Merino R., and Nuñez G. (1995). Bcl-x$_L$ displays restricted distribution during T-cell development and inhibits multiple forms of apoptosis but not clonal deletion in transgenic mice. J. Exp. Med., in press.
12. Fernandez-Sarabia, M. and Bischoff, J.R. (1993). Bcl-2 associates with the *ras*-related protein R-*ras* p23. Nature, 366:274-275.
13. Oltvai, Z.N., Milliman, C.L., and Korsmeyer, S.J. (1993). Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. Cell, 74:609-619.
14. Yang, E., Zha, J., Jockel, J., Boise, L.H., Thompson, C.B. and Korsmeyer, S.J. (1995). Bad, a heterodimeric partner for Bcl-x$_L$ and Bcl-2, displaces Bax and promotes cell death. Cell, 80:285-291.
15. Chittenden, T., Harrington, E.A., O'Connor, R., Flemington, C., Lutz, R.J., Evan, G.I. and Guild, B.C. (1995). Induction of apoptosis by the Bcl-2 homologue Bak. Nature, 374:733-736.
16. Boyd, J. M., Malstrom, S., Subramanian, T., Venkatesh, L. K., Schaeper, U., Elangovan, B., D'Sa-Eipper, C., and Chinnadural, G. (1994). Adenovirus E1B and 19 kDa and Bcl-2 proteins interact with a common set of cellular proteins. Cell, 79:341-351.

17. Takayama, S., Sato, T., Krajewski, S., Kochel, K., Irie, S., Millan, J.A. and Reed, J.C. (1995). Cloning and functional analysis of BAG-1: a novel Bcl-2-binding protein with anti-cell death activity. Cell, 80:279-284.
18. Nuñez G. and Clarke M. F. (1994). The Bcl-2 famly of proteins: regulators of cell death and survival. Trends Cell. Biol., 4:399-403.
19. Campos, L., Rouault, J.-P., Sabido, O., Oriol, P., Roubi, N., Vasselon, C., Archimbaud, E., Magaud, J.-P., and Guyotat, D. (1993). High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. Blood. 81:3091-3096.
20. Castle, V.P., Heidelberger, K.P., Bromberg, J., Ou, X., Dole, M., and Nuñez, G. (1993). Expression of the apoptosis-suppressing protein Bcl-2 in neuroblastoma is associated with poor stage disease, unfavorable histology and N-*myc* amplification. Am. J. Pathol., 143:1543-1550.
21. Colombel, M., Symmans, F., Gil, S., O'Toole, K.M., Chopin, D., Benson, M., Olsson, C.A., Korsmeyer, S., and Buttyan, R. (1993). Detection of the apoptosis-suppressing oncoprotein bcl-2 in hormone-refractory human prostate cancers. Am. J. Pathol., 143:390-400.
22. Silvestrini, R., Veneroni, S., Daidone, M.G., Benini, E., Boracchi, P., Mezzetti, M., Di Fronzo, G., Rilke, F., and Veronesi, U. (1994). The Bcl-2 protein: a pronostic indicator strongly related to p53 protein in lymph node-negative breast cancer patients. J. Natl. Cancer Inst., 86:499-504.
23. Dole, M.G., Jasty, R., Cooper, M.J., Thompson, C.B., Nunez, G. and Castle, V. P. (1995). Bcl-x_L is expressed in neuroblastoma cells and modulates chemotherapy-induced apoptosis. Cancer Res., 55: 2576-2582.
24. Clarke, M. F., Apel I. J., Benedict M. A., Eipers P. J., Sumantran V., González-García M., Doedens M., Fukunaga N., Davidson B., Dick J. E., Minn A. J., Boise L. H., Thompson C. B., Wicha M., and Núñez G. (1995). A recombinant *bcl-*x_S adenovirus selectively induces apoptosis in cancer cells, but not normal bone marrow cells. Proc. Natl. Acad. Sci. USA 92,11024-11028.
25. Boise, L.H., González-Garcia, M., Postema, C.E., Ding, L., Lindsten, T., Turka, L.A., Mao, X., Nuñez, G., and Thompson, C.B. (1993). *bcl-x*, a *bcl-2* related gene that functions as a dominant regulator of apoptotic cell death. Cell, 74:597-608.
26. Chinnaiyan AM, O'Rourke K, Lane BR, and Dixit VM. Interaction of CED-4 with CED-3 and CED-9: A molecular framework for Cell Death. (1997). Science 275:1122-1126.
27. Wu, D., Wallen H., Nuñez G. Interaction and regulation of subcellular localization of CED-4 by CED-9. (1997). Science 275:1126-1128.
28. Chang BS, Minn AJ, Muchmore SW, Fesik SW, Thompson CB. Identification of a novel regulatory domain in Bcl-X(L) and Bcl-2. The EMBO J 16(5), 968-977 (1997).